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Regulation of Bone Sialoprotein (BSP) Gene Transcription by Lipopolysaccharide

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Abstract Lipopolysaccharide (LPS) is a major mediator of inflammatory responses in periodontal disease that inhibits bone formation and stimulates bone resorption. To determine the molecular mechanisms involved in the suppression of bone formation, we have analyzed the effects of LPS on BSP gene expression. Bone sialoprotein (BSP) is a mineralized tissue-specific protein that appears to function in the initial mineralization of bone. Treatment of osteoblast-like ROS 17/2.8 cells with LPS (1 μg/ml) for 12 h caused a marked reduction in BSP mRNA levels. The addition of antioxidant *N*-acetylcysteine (NAC; 20 mM) 30 min prior to stimulation with LPS attenuated the inhibition of BSP mRNA levels. Transient transfection analyses, using chimeric constructs of the rat BSP gene promoter linked to a luciferase reporter gene, revealed that LPS (1 μg/ml) suppressed expression of luciferase construct, encompassing BSP promoter nucleotides −108 to +60, transfected into ROS17/2.8 cells. The effects of LPS were inhibited by protein kinase A (PKA) inhibitor, H89 and the tyrosine kinase inhibitor, herbimycin A (HA). Introduction of 2 bp mutations in the inverted CCAAT box (ATTGG; nts −50 and −46), a cAMP response element (CRE; nts −75 to −68), a FGF response element (FRE; nts −92 to −85), and a pituitary specific transcription factor binding element (Pit-1; nts −111 to −105) showed that the LPS effects were mediated by the CRE and FRE. Whereas the FRE and 3′-FRE DNA-protein complexes were decreased by LPS, CRE DNA-protein complex did not change after LPS treatment. These studies, therefore, show that LPS suppresses BSP gene transcription through PKA and tyrosine kinase-dependent pathways and that the LPS effects are mediated through CRE and FRE elements in the proximal BSP gene promoter. J. Cell. Biochem. 97: 368–379, 2006.

Key words: bone sialoprotein; lipopolysaccharide; gene regulation; mineralized tissues; transcription

Lipopolysaccharide (LPS) is sufficient to induce immune and inflammatory responses, and increases production of prostaglandin E_2 (PGE_2) and cytokines in macrophages, lymphocytes, and endothelial cells the same as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) [Lynn and Golenbock, 1992]. PGE_2 and these cytokines activate immune systems to defend the host from bacterial infection [Schletter et al., 1995]. Recently, mouse toll-like receptor 4 (TLR4) was identified as the receptor for LPS [Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999]. The intercellular signaling pathway of TLR4 is similar to that of IL-1 receptors [O'Neill and Greene, 1998]. Both receptors have been shown to use TNF receptor-associated factor 6 (TLR6) as a common signaling molecule [Zhang et al., 1999]. LPS and IL-1 induce the activation of NF-κB, which regulates the expression of many genes involved in immune and inflammatory responses [Zhang et al., 1999].

Bone sialoprotein (BSP) is a mineralized tissue-specific noncollagenous protein that is

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glycosylated, phosphorylated, and sulfated [Oldberg et al., 1988; Ogata et al., 1995]. The temporo-spatial deposition of BSP into the extracellular matrix of bone [Chen et al., 1992] and the ability of BSP to nucleate hydroxyapatite crystal formation [Hunter and Goldberg, 1993; Ganss et al., 1999] indicate a potential role for BSP in the initial mineralization of bone and cementum. BSP is also expressed in breast, lung, thyroid, and prostate cancers [Ibrahim et al., 2000; Waltregny et al., 2000]. Thus, it has been suggested that BSP may be involved in the osteotropism of metastatic cancer cells through its ability to bind to hydroxyapatite crystals and to mediate cell attachment through cell-surface integrin [Ganss et al., 1999]. Therefore, regulation of the BSP gene is potentially important in the differentiation of osteoblasts, in bone matrix mineralization, and in tumor metastasis. There are several studies concerning about the transcriptional regulation of BSP gene expression, and *cis*-acting elements on BSP gene promoters [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999]. In the BSP promoter, there is an inverted TATA element [Li et al., 1995] overlapping a vitamin D response element [Kim et al., 1996] and an inverted CCAAT box (−50 to −46), which is required for basal transcriptional activity [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, cAMP response element (CRE) [Samoto et al., 2002, 2003], a fibroblast growth factor 2 response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Shimizu et al., 2004a, 2005], a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105) that mediates the stimulatory effects of parathyroid hormone [Ogata et al., 2000; Shimizu et al., 2004a], and a homeodomain binding element (HOX; −199 to −192) [Benson et al., 2000; Shimizu et al., 2004b], have been characterized. Further upstream in the rat promoter, a transforming growth factor-β activation element (−499 to −485) [Ogata et al., 1997; Shimizu et al., 2004b, 2005], and a glucocorticoid response element (−920 to −906), overlapping an AP-1 site (−921 to −915) [Ogata et al., 1995; Yamauchi et al., 1996] have also been identified.

Periodontitis is a chronic inflammatory disease characterized by gingival inflammation and alveolar bone resorption [Soolari et al., 1999]. It is often caused by infections with Gram-negative bacteria, such as *Porphyromonas gingivalis (P. gingivalis)* [Haapasalo et al., 1986; Slots, 1986] and *Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans)* [Zambon et al., 1983, 1986], and LPS from Gram-negative bacteria has been identified as an important factor in the pathogenesis [Daly et al., 1980]. LPS is a complex glycolipid composed of a hydrophilic polysaccharide protein and hydrophobic domain known as lipid A that is responsible for most of the LPS-induced biological effects [Takada and Kotani, 1989]. One of the function of LPS is induction of osteoclast differentiation and stimulation of bone resorption [Amano et al., 1997]. Further, LPS increases IL-1, IL-6, and PGE_2 production in osteoblasts, and these cytokines might be involved in LPS-mediated periodontitis [Nair et al., 1996].

To elucidate molecular pathways through which LPS suppresses bone formation, we have analyzed the effects of LPS on BSP gene expression in ROS 17/2.8 cells. These studies show that LPS suppresses transcription of the BSP gene through PKA and tyrosine kinase pathways and that the effects are mediated through CRE and FRE in the proximal promoter of BSP gene.

MATERIALS AND METHODS

Materials

α-minimum essential medium (α-MEM), fetal calf serum (FCS), Lipofectamine, penicillin, and streptomycin, SuperScript one step RT-PCR with Platinum Taq and Trypsin were obtained from Invitrogen (Carlsbad, CA). The pGL3-basic vector and pSV-β-galactosidase control vector were purchased from Promega Co., (Madison, WI). LPS (L2630, *Escherichia coli* 0111: B4), *N*-Acetylcysteine (NAC), and forskolin (FSK) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Recombinant human FGF2 was from Genzyme Techne (Minneapolis, MN). The protein kinase inhibitors, H89 and H7 were from Seikagaku Corporation (Tokyo, Japan), and the tyrosine kinase inhibitor, herbimycin A (HA) and guanidium thiocyanate were purchased from Wako Pure Chemical (Tokyo, Japan).

Cell Cultures

Rat clonal osteoblast cell line, ROS17/2.8 cells were used in this study [Majeska et al., 1980]. Rat stromal bone marrow cells (SBMC) [Pitaru

et al., 1993], were kindly provided by Dr. S. Pitaru (Tel Aviv University, Israel). Cells were first grown to confluence in 60 mm dishes in α-MEM with 10% FCS and change to serum free α-MEM and incubated with LPS (1 μg/ml) for 12 h. Total RNA was isolated from triplicate cultures and analyzed for the expression of BSPmRNA by Northern hybridization as described below.

Northern Hybridization

Twenty microgram aliquots of total RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond N+ membrane. Hybridizations were performed at 42°C with either ^{32}P-labeled rat BSP, osteopontin (OPN), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Following hybridization, membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate, pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6 and 2.0 kb) of rat BSPmRNA, were scanned in a Bio-imaging analyzer (BAS2000, Fuji Film, Tokyo, Japan).

RT-PCR

Following treatments by LPS, NAC, and LPS+NAC for 12 h total RNA was extracted from ROS17/2.8 cells with guanidium thiocyanate, and 0.1 μg was used as a template for SuperScript one-step RT-PCR. Primers were synthesized on the basis of the reported rat cDNA sequences for BSP [Oldberg et al., 1988] and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Sequences of the primers used for RT-PCR and the expected size of the PCR products were as follows: BSP forward, 5′-CTGCTTTAATCTTGCTCTG-3′; BSP reverse, 5′-CCATCTCCATTTTCTTCC-3′ (211 bp); GAPDH forward, 5′- CCATGTTTG-TGATGGGTGTG -3′; GAPDH reverse, 5′-GGATGCAGGGATGATGTTCT- 3′ (264 bp). cDNA synthesis and pre-denaturation were performed for 1 cycle at 50°C, 30 min; 94°C, 2 min, and amplification was carried out for 40 cycles (BSP) and 30 cycles (GAPDH) at 94°C, 30 s; 55°, 30 s; 72°C, 30 s, and final extension at 72°C, 10 min in a 50 μl reaction mixture. After amplification, each reaction mixture was analyzed by 2 % agarose gel electrophoresis, and the bands were visualized by ethidium bromide staining.

Transient Transfection Assay

Exponentially growing ROS17/2.8 cells were used for transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a Lipofectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct [Ogata et al., 1995] and 2 μg pSV-β-galactosidase (β-gal) vector as an internal control. Two days post-transfection, cells were deprived of serum for 12 h, each test compound was added, and the cells were cultured for a further 12 h prior to harvesting. The luciferase assays were performed according to the supplier's protocol (PicaGene, Toyo Inki, Tokyo, Japan) using a Luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan) to measure the luciferase activities. The protein kinase inhibitor, H89 (5 μM) and H7 (5 μM) were used to inhibit protein kinase A (PKA) and protein kinase C (PKC), respectively. HA (1 μM) was used as the tyrosine kinase inhibitor.

Gel Mobility Shift Assay

Confluent ROS17/2.8 cells in T-75 flasks incubated for 12 h with LPS (1 μg/ml) in α-MEM without serum were used to prepare the nuclear extracts as described previously [Dignam et al., 1983]. Double-stranded oligonucleotides encompassing an inverted CCAAT (nts −61 to −37, 5′-CCGTGACCGTGATTGGCTGCTGAGA −3′), a CRE (nts −84 to −59, 5′-CCCACAGCCTGA-CGTCGCACCGGCCG −3′), a FRE (nts −98 to −79, 5′-TTTTCTGGTGAGAACCCACA −3′), a 5′-FRE, which include a putative NFκB sequence (nts −112 to −93, 5′-GTTGTAGTTACG-GATTTTCT −3′), and a 3′-FRE (nts −95 to −73, 5′-TCTGGTGAGAACCCACAGCCTGA −3′) in the BSP promoter (Fig. 5) were prepared by Bio-Synthesis, Inc. (Lewisville, TX). For gel shift assay, double-stranded oligonucleotides were end labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (RT = 21°C) with 0.1 pM radiolabeled double-stranded oligonucleotides in buffer containing 50 nM KCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.9), 1 mM DTT, 0.04% Nonidet P-40, 5% glycerol and 1 μg poly (dI-dC). Following incubation, the protein-DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels (38:2 acrylamide/bisacrylamide) run at 200V at RT. Following electrophoresis, the gels were dried and

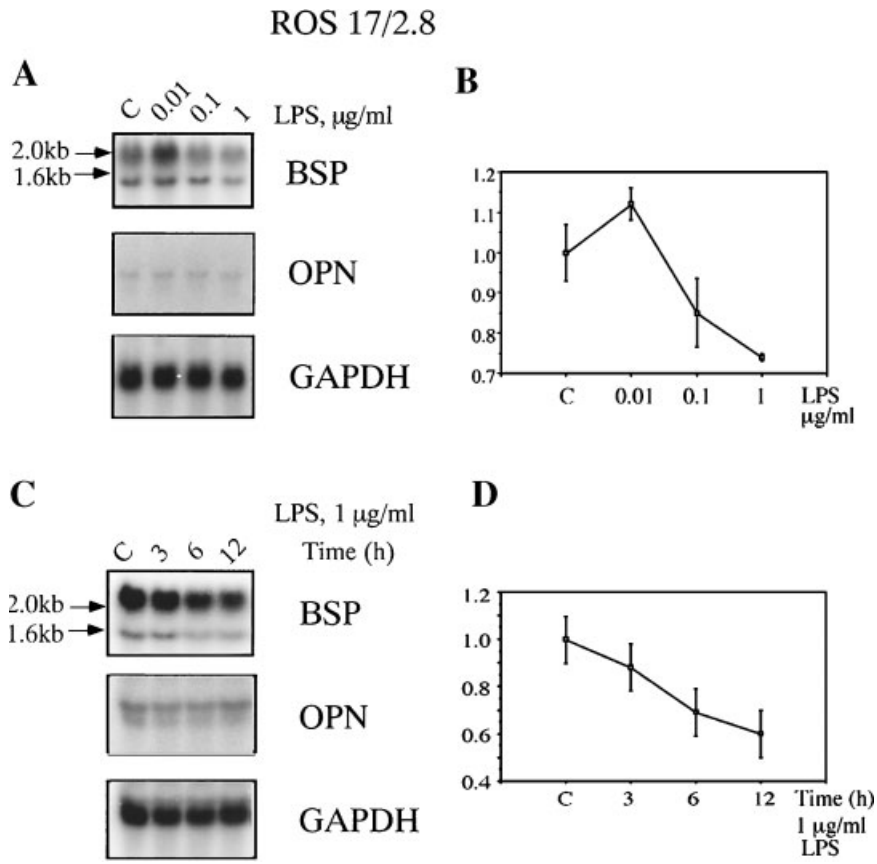

Fig. 1. Northern hybridization analysis of LPS effects on BSPmRNA levels in ROS17/2.8 Cells. **A**: Dose response of the effect of LPS on BSPmRNA levels in ROS17/2.8 cells treated for 12 h. At 0.01–1 μg/ml, LPS decreased BSPmRNA level maximal at 1 μg/ml LPS. **B**: Quantitative analyses of the triplicate data sets are shown with error bars. **C**: A 12 h time course revealed a decrease in BSPmRNA level following treatment of 1 μg/ml LPS. **D**: Quantitative analyses of the triplicate data sets are shown with error bars. Total RNA was isolated from triplicate cultures harvested after incubation for 3, 6, and 12 h and used for Northern hybridization. In addition, results of hybridization using osteopontin (OPN) probe on the same filters are shown for comparison.

autoradiograms prepared and analyzed using an image analyzer (BAS2000, Fuji film, Tokyo, Japan).

Statistical Analysis

Significant differences between control and treatment were determined using Student's *t*-test.

RESULTS

Suppression of BSP mRNA Expression in ROS 17/2.8 Cells

To study the regulation of BSP expression by LPS, we used ROS 17/2.8 cells, which have been shown to have osteoblastic characteristics and to constitutively express BSP mRNA [Ogata et al., 1995]. First, a dose–response effect of LPS

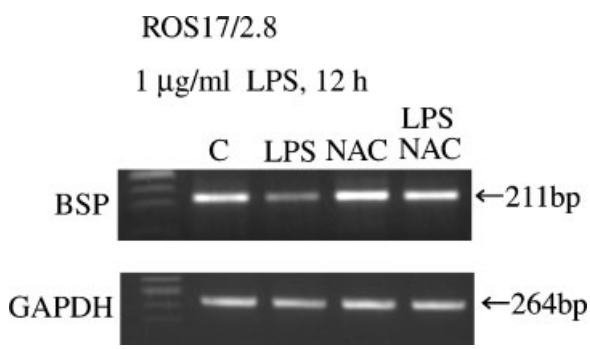

Fig. 2. Effect of antioxidant on the suppressive effect of LPS in ROS17/2.8 cells. The cells were treated with 20 mM *N*-acetylcystein (NAC) for 30 min and then incubated with LPS together with NAC for 12 h. NAC markedly attenuated the LPS-dependent suppression of BSP mRNA.

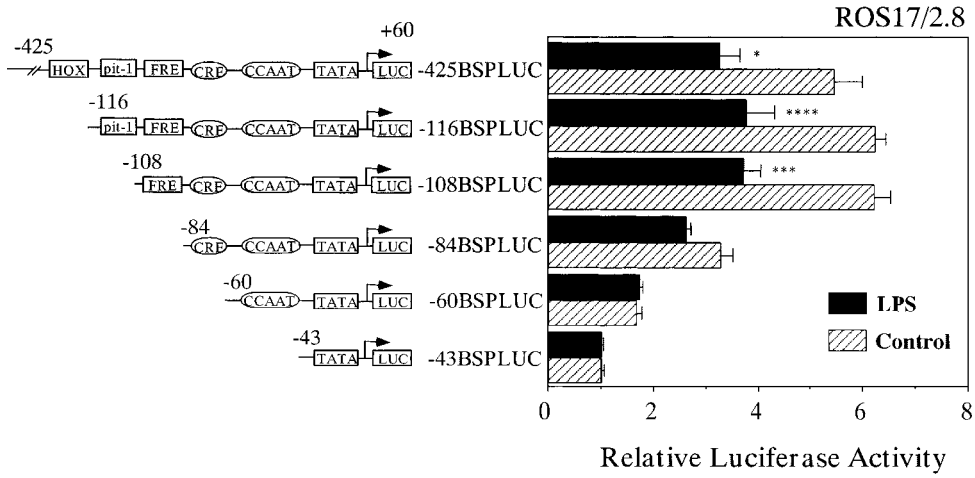

Fig. 3. LPS suppresses BSP promoter activity in ROS 17.2.8 cells. Transient transfection of chimeric constructs encompassing different regions of the rat BSP gene promoter ligated to a luciferase reporter gene, were performed in ROS17/2.8 cells. The results of transfection assays indicated a decrease in transcription after 12 h treatment by 1 μg/ml LPS using −43, −60, −84, −108, −116, and −425 BSPLUC constructs. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values are expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: $^{*}P < 0.1$; $^{***}P < 0.02$; $^{****}P < 0.01$.

on BSP expression was established by treating the ROS 17/2.8 cells with different concentrations of LPS for 12 h and measuring the BSP mRNA levels by Northern blot analysis. Whereas 0.01 μg/ml LPS increased the BSP mRNA level, 0.1 and 1 μg/ml LPS decreased BSP mRNA levels with a maximal effect at 1 μg/ml LPS (Fig. 1A,B). In addition, results of hybridization analyses for OPN and GAPDH on the same samples are shown for comparison (Fig. 1A). Thus, 1 μg/ml of LPS was used to determine the time course of BSP mRNA

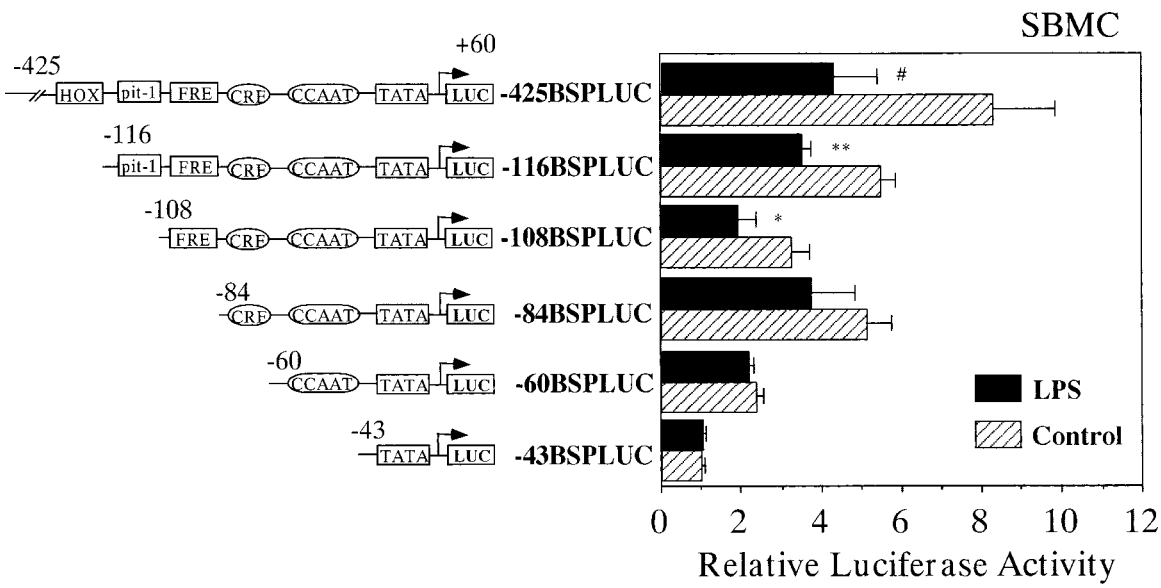

Fig. 4. LPS suppresses BSP promoter activity in stromal bone marrow cells. Transient transfection of chimeric constructs encompassing different regions of the rat BSP gene promoter ligated to a luciferase reporter gene were performed in stromal bone marrow (SBMC) cells. The results of transfection assays indicated a decrease in transcription after 12 h treatment by 1 μg/ml LPS using −43, −60, −84, −108, −116, and −425 BSPLUC constructs. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: $^{\#}P < 0.2$; $^{*}P < 0.1$; $^{**}P < 0.05$.

expression. LPS suppressed BSP mRNA level at 12 h (Fig. 1C,D), whereas no effects on OPN and GAPDH mRNA were observed (Fig. 1C).

Effect of Antioxidant on the Suppressive Effect of LPS

We previously reported the inhibitory effects of TNF-α on the BSP promoter activity, which is mediated by CRE [Samoto et al., 2002]. This points to the possibility that LPS effects on the BSP promoter could be mediated by TNF-α secreted in response to LPS. To address this question, we analyzed the effects of an antioxidant (*N*-acetylcysteine, NAC) on the suppressive effect of LPS. Since TNF-α is known to stimulate reactive oxygen species, ROS 17/2.8 cells were treated with 20 mM NAC for 30 min and then incubated with LPS together with NAC for 12 h. NAC markedly attenuated the LPS-dependent suppression of BSP mRNA (Fig. 2).

Transient Transfection Analysis of Rat BSP Promoter Constructs

To determine how LPS-regulates BSP expression, sequences from the 5′-flanking region of the BSP gene were tested for transcriptional activity and responsiveness to LPS by transient transfection of chimeric constructs in ROS 17/2.8 cells. The constructs −43BSPLUC (−43 to +60), −60BSPLUC (−60 to +60), −84BSPLUC (−84 to +60), −108BSPLUC (−108 to +60), −116BSPLUC (−116 to +60), and −425BSPLUC (−425 to +60) and their responsiveness to LPS (1 μg/ml) are shown in Figure 3. The transcriptional activity of −108BSPLUC, which encompasses nucleotides −108 to +60, was reduced ∼0.59-fold and almost the same level of decrease was observed in constructs −116BSPLUC and −425BSPLUC. However, deletion of the sequence between −108 and −84 abolished the LPS-mediated reduction. When

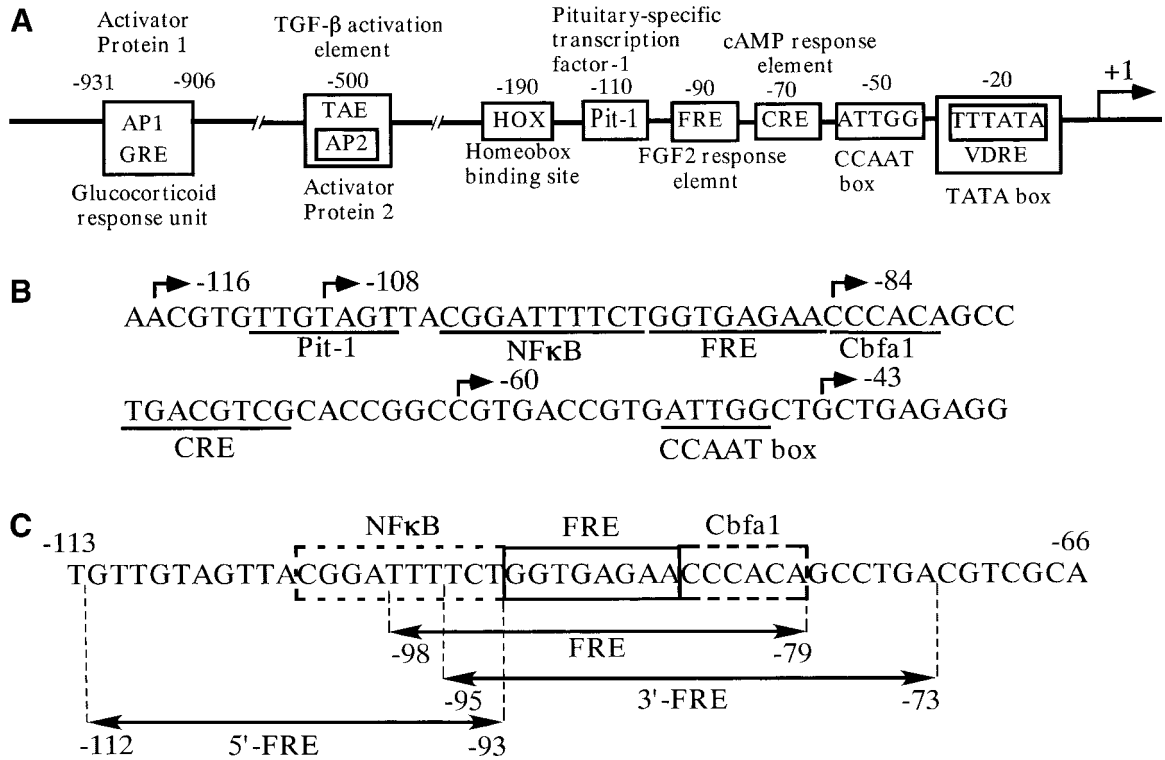

Fig. 5. Regulatory elements in the proximal promoter of rat BSP gene. **A**: The positions of the inverted TATA and CCAAT boxes, vitamin D response element (VDRE) that overlaps the inverted TATA box, a CRE, a FRE, Pit-1, homeobox-binding site (HOX), TGF-β activation element (TAE) overlaps with AP2 and glucocorticoid response element overlapping with AP1 are shown in the proximal promoter region of the rat BSP gene. **B**: The nucleotide sequence of the rat BSP gene proximal promoter is shown from nts −116 to −43. An inverted CCAAT box (nts −50 ∼ −46), a CRE (nts −75 ∼ −68), a putative Cbfa1 (nts −84 ∼ −79), a FRE (nts −92 ∼ −85), a NFκB (nts −102 ∼ −93), and a Pit-1 (nts −111 ∼ −105) are present. **C**: Nucleotide sequences of oligonucleotide probes used for gel mobility shift assays. The sequence of the rat BSP promoter region is shown to identify the location of the oligonucleotide probes that were used in gel mobility shift assays. Dotted boxes denote the position of the NFκB and Cbfa1, and a solid box indicates FRE.

transcriptional activity in response to LPS was analyzed in normal rat stromal bone marrow cells (SBMC), the transcriptional activities of −108BSPLUC (~0.59-fold), −116BSPLUC (~0.64-fold), and −425BSPLUC (~0.52-fold) were decreased by LPS treatment (Fig. 4). Within the DNA sequence that is unique to −108BSPLUC (between nts −108 and −84), a putative NFκB (nts −102 and −93) and FGF2 response element (FRE; nts −92 and −85) are present (Fig. 5).

Since LPS signaling is mediated by protein kinases, we investigated the effects of the PKC inhibitor, H7 (5 μM), the PKA inhibitor, H89 (5 μM), and the tyrosine kinase inhibitor, HA (1 μM) on LPS-mediated transcription. Whereas LPS suppression of −116BSPLUC promoter activity was inhibited by H89 and HA, no effect was observed for H7 (Fig. 6), indicating an involvement of PKA and tyrosine kinase in the signaling pathway. FSK- (1 μM, PKA activator) and FGF2- (10 ng/ml, activator of tyrosine kinase pathway) stimulated BSP promoter activities were both inhibited by LPS (Fig. 7).

Next, we introduced two base pair mutations in the −116BSPLUC. Whereas mutations in the Pit-1 (M-PIT; TTacAGT) had no effect on LPS suppression and mutation of the CCAAT box (M-CCAAT; ATTtt) essentially abolished basal expression, mutations of the CRE (M-CRE; cGACGcCG), and FRE (M-FRE; GGcaAGAA) significantly reduced the LPS effects on the transcriptional activities (Fig. 8).

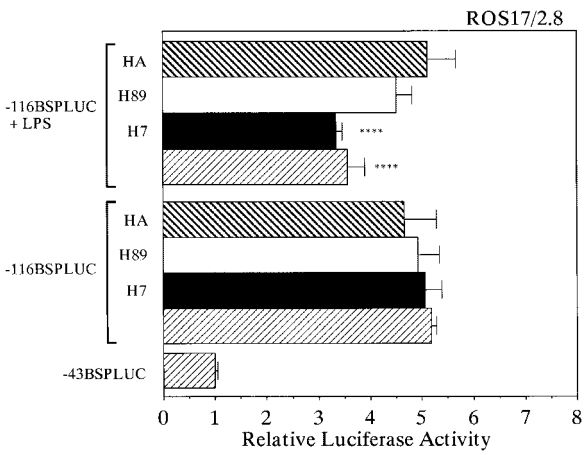

Fig. 6. Effect of kinase inhibitors on the inhibitory effects on BSP transcription. Transient transfection analyses of −116BSPLUC construct in the presence or absence of LPS (1 μg/ml) in ROS 17/2.8 cells are shown together with the effects of the PKC inhibitor (H7, 5 μM), PKA inhibitor (H89, 5 μM) and tyrosine kinase inhibitor (HA, 1 μM). The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: $^{****}P < 0.01$.

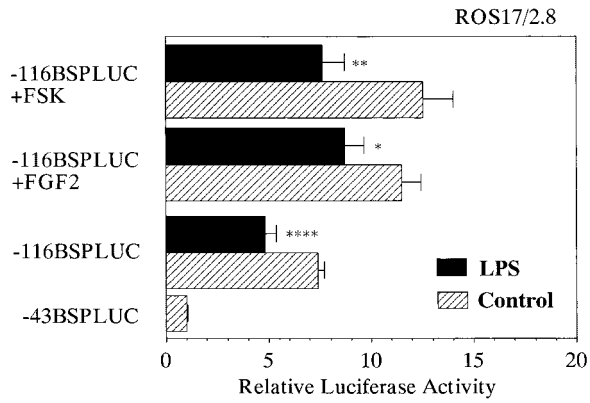

Fig. 7. Effect of LPS on the BSP promoter activity in the presence of forskolin and FGF2. Transient transfection analyses of −116BSPLUC construct in the presence or absence of LPS (1 μg/ml) in ROS 17/2.8 cells are shown together with the effects of forskolin (FSK, 1 μM) and FGF2 (10 ng/ml). The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: $^{*}P < 0.1$, $^{**}P < 0.05$, $^{****}P < 0.01$.

Gel Mobility Shift Assays

To clarify which nuclear proteins bind to the CRE and FRE and mediate the LPS effects on transcription, double-stranded oligonucleotides were end-labeled and incubated with equal amount (3 μg) of nuclear proteins extracted from confluent ROS17/2.8 cells that were either not treated (control) or treated with 1 μg/ml LPS for 3, 6, and 12 h. When we used the inverted CCAAT sequence as a probe, the DNA-NF-Y protein complex [Tezuka et al., 1996; Kim and Sodek, 1999; Shimizu and Ogata, 2002] did not change after LPS treatment (Fig. 9, lanes 1–4). When the FRE and 3′-FRE were used as probes, the formation of FRE DNA-protein complexes (Fig. 9, lanes 5–8) and rapidly migrating 3′-FRE DNA-protein complexes (Fig. 9, lanes 9–12) were decreased by LPS. In comparison, 5′-FRE (including a putative NFκB) DNA-protein complexes (Fig. 10, lanes 1–4) did not change after LPS stimulation. In a previous study, we identified CRE DNA-protein complex as a CRE-binding protein (CREB) [Samoto et al., 2003]. When the CRE was used as a probe, CREB binding was unaffected following stimulation by LPS (Fig. 10, lanes 5–8). A faster migrating band is present only in the nuclear extracts of

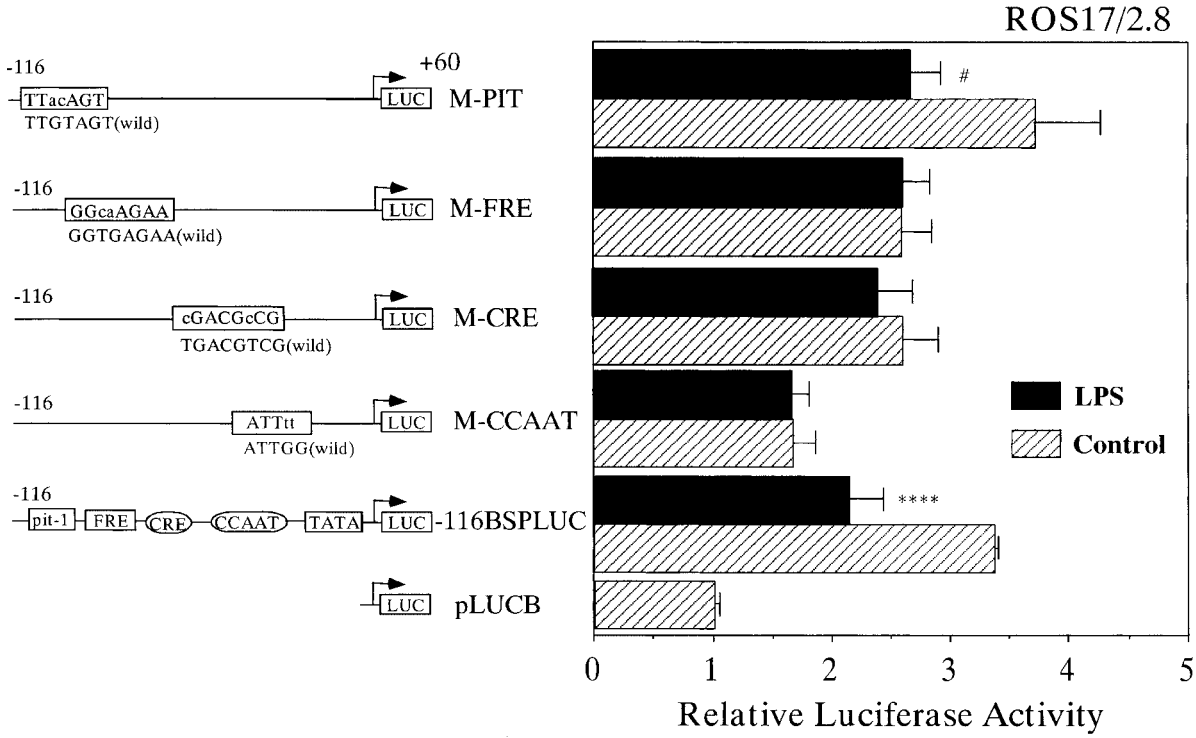

Fig. 8. Site mutation analysis of LUC activities in response to LPS. Dinucleotide substitutions were made within the context of the homologous −116 to +60 BSP promoter fragment (−116BSPLUC). M-CCAAT (ATTtt), M-CRE (cGACGcCG), M-FRE (GGcaAGAA), and M-PIT (TTacAGT) constructs were analyzed for relative promoter for inhibition in the presence of LPS (1 μg/ml) for 12 h. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences compared to controls are shown at the following probability levels: $^{\#}P < 0.2$; $^{****}P < 0.01$.

LPS treated cells. That the DNA-protein complex represents a specific interaction was indicated by competition experiments in which an excess (20- and 40-fold) of CRE reduced the amount of complex formation. In contrast, a faster migrating band did not compete with complex formation (Fig. 10, lanes 9 and 10). The results suggest the faster migrating complex of the EMSA is non-specific.

DISCUSSION

These studies show that LPS suppresses the transcription of BSP, a major protein of the bone matrix. Treatment of ROS 17/2.8 cells with LPS decreased the steady-state level of BSP mRNA (Fig. 1), reflecting a reduction of gene transcription measured by transient transfection assays. Furthermore, an antioxidant NAC abolished the suppressive effect of LPS. These suggest LPS effect might be mediated through TNF-α, since TNF-α is known to stimulate reactive oxygen species. The effects of LPS were mediated by PKA and tyrosine kinase activities (Fig. 6). FSK- and FGF2-induced BSP promoter activities were inhibited by LPS (Fig. 7). The results show that bidirectional role of cAMP and tyrosine kinase pathways in the BSP promoter. From the transient transcription assays the sites of transcriptional regulation by LPS could be identified as CRE and FRE elements in the proximal promoter of the BSP gene. Although CREB binding to CRE did not change after LPS treatment (Fig. 10, lanes 5–8). PKA signaling does not affect CREB binding to its cognate CRE element, it can direct phosphorylation of CREB, which is required for transcriptional regulation [Lee et al., 1990; Pearman et al., 1996; Samoto et al., 2003]. In comparison, the nuclear factor binding to the FRE, which is regulated by tyrosine kinase [Shimizu-Sasaki et al., 2001], suppressed by LPS (Fig. 9, lanes 5–8). Binding activities of nuclear proteins to 3′-FRE was also suppressed by LPS (Fig. 9, lanes 9–12). Previously, we identified a FRE (GGTGAGAA) in the rat BSP

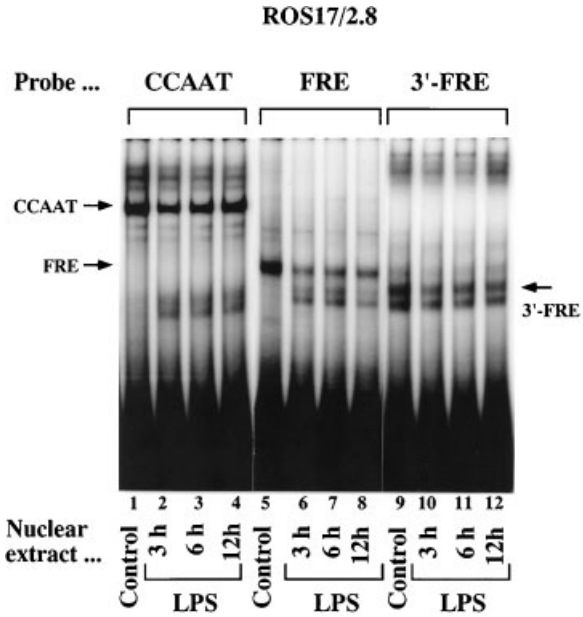

Fig. 9. Gel mobility shift assays using inverted CCAAT, CRE, and FRE. Radiolabeled double-stranded inverted CCAAT (-61 CCGTGACCGTGATTGGCTGCTGAGA -37), FRE (-98 TTTTCTGGTGAGAACCCACA -79), and 3′-FRE (-98 TCTGGTGAGAACCCACAGCCTGA -79) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells incubated without (**lanes 1**, **5**, and **9**) or with LPS (1 μg/ml) for 3 h (**lanes 2**, **6**, and **10**), and 6 h (**lanes 3**, **7**, and **11**) and 12 h (**lanes 4**, **8**, and **12**). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using a bioimaging analyzer.

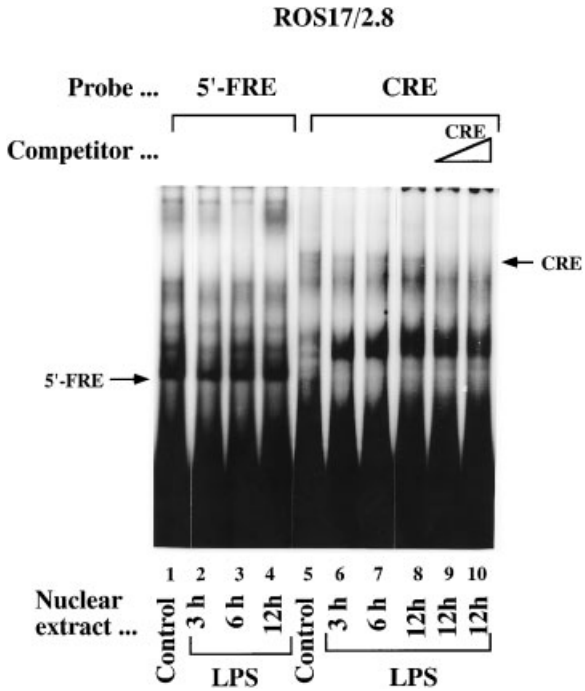

Fig. 10. Radiolabeled double-stranded CRE (-84 CCCACAGCC**TGACGTCG**CACCGGCCG -59), and 5′-FRE (-112 GTTGTAGTTA**CGGATTTTCT** -93) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells incubated without (**lanes 1** and **5**) or with LPS (1 μg/ml) for 3 h (**lanes 2** and **6**), and 6 h (**lanes 3**, **7**) and 12 h (**lanes 4**, **8, 9, 10**). Competition reactions were performed using a 20- and 40-fold molar excess unlabeled CRE (**lanes 9** and **10**). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using a bioimaging analyzer.

proximal gene promoter [Shimizu-Sasaki et al., 2001] that is juxtaposed to a putative Cbfa1 binding site (CCCACA; Osf2, Runx2) (Fig. 5) [Ducy and Karsenty, 1995; Komori et al., 1997]. Cbfa1 plays an essential role in osteoblast differentiation. Cbfa1-deficient mice display complete absence of bone due to arrested osteoblast differentiation [Komori et al., 1997]. 3′-FRE has a putative Cbfa1 site in the middle of the sequence. The FRE and Cbfa1 sequences are closely spaced in the BSP promoter, and mutation of the Cbfa1 sequence induced transcriptional activity (unpublished results), showing that the FRE-binding protein and Cbfa1 might be interacting with each other. Further study is necessary to elucidate the interaction of these transcription factors.

When we used SBMC cells for luciferase assays to see the effect of LPS on BSP transcription, LPS suppressed BSP transcription (Fig. 4). Therefore, LPS suppresses BSP expression not only in transformed ROS 17/2.8 cells but also in normal osteoprogenitor cells. −108BSPLUC activity was lower than those from −84BSPLUC and −116BSPLUC in SBMC cells. Further study is necessary to resolve the reason.

LPS is a major mediator of inflammatory responses in periodontal diseases [Daly et al., 1980; Yoshimura et al., 1997; Soolari et al., 1999]. LPS decreases the synthesis of bone matrix protein and stimulates bone resorption [Nair et al., 1996; Amano et al., 1997]. LPS from periodontopathic bacteria induced secretion of inflammatory cytokines by human polymorphonuclear leukocytes [Yoshimura et al., 1997]. *P. gingivalis* LPS induces intercellular adhesion molecule-1 (ICAM-1) expression in human

gingival fibroblasts in a soluble CD14 dependent manner [Masaka et al., 1999]. These findings suggest that LPS is one of the crucial factors in periodontal diseases. In this study, we used LPS derived from *Escherichia coli* (*E. coli*). However, periodontopathic bacteria such as *P. gingivalis* and *A. actinomycetemcomitans* derived LPS have different characteristics [Nair et al., 1983; Yoshimura et al., 1997]. Therefore, in the next step, we wish to determine the effects of LPS from periodontopathic bacteria on BSP gene expression.

These studies show that LPS suppresses BSP transcription mediated through CRE and FRE elements in the proximal promoter of the BSP gene. In summary, we have shown that CRE and FRE exist in the rat BSP proximal promoter through which the inhibitory effects of LPS on BSP gene transcription are mediated. Since BSP is expressed by differentiated osteoblasts, and LPS is one of the virulent factors for periodontitis and stimulates bone resorption, it is conceivable that CRE and FRE-binding transcription factors may contribute to LPS-mediated inflammatory responses and inhibitory effects on BSP expression.

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